Myrothecium roridum Tri4 encodes a multifunctional oxygenase required for three oxygenation steps

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Abstract: The biosyntheses of both macrocyclic trichothecenes in *Myrothecium roridum* and simple trichothecenes in *Fusarium* species begin with the cyclization of farnesyl pyrophosphate to form the sesquiterpene hydrocarbon trichodiene. A previous study showed that *Myrothecium* has a cluster of 3 genes that are homologous with *Fusarium* trichothecene genes: *Tri4*, a P450 oxygenase; *Tri5*, the sesquiterpene cyclase; and *Tri6*, a zinc-finger regulatory gene. *Fusarium graminearum Tri4* (*FgTri4*) and *M. roridum MrTri4* (*MrTri4*) have 66.9% identity. In this study, *MrTri4* was expressed in *Fusarium verticillioides*. Liquid cultures of transformant strains expressing *MrTri4* converted exogenous trichodiene to isotrichodiol, indicating that *MrTri4* controls 3 oxygenation steps and that the product of MrTRI4 is isotrichodiol.

Key words: trichothecene, P450 oxygenase, trichodiene, *Tri4*, multifunctional oxygenase, mono-oxygenase, isotrichodiol, 12,13-epoxytrichothec-9-ene.

Résumé : La biosynthèse de trichotécènes macrocycliques chez *Myrothecium roridum* et de trichothécènes simples chez *Fusarium* commence par la cyclisation du farnésyl pyrophosphate afin de former l'hydrocarbure de sesquiterpène trichodiène. Une étude précédente avait montré que *Myrothecium* possédait une grappe de 3 gènes homologues aux gènes de *Fusarium* : *Tri4*, une P450 oxygénase; *Tri5*, une sesquiterpène cyclase; et *Tri6*, un gène régulateur à doigts de zinc. Les gènes *Tri4* de *Fusarium graminearum* (*FgTri4*) et de *M. roridum* (*MrTri4*) possèdent 66,9 % d'identité. Dans cette étude, *MrTri4* a été exprimé chez *Fusarium verticillioides*. Des cultures liquides des souches transformantes exprimant *MrTri4* convertissaient le trichodiène exogène en isotrichodiol, indiquant que *MrTri4* contrôle 3 étapes d'oxygénation et que le produit de MrTRI4 est l'isotrichodiol.

Mots-clés : trichothécène, P450 oxygénase, trichodiène, *Tri4*, oxygénase multi-fonctionnelle, mono-oxygénase, isotrichodiol, 12,13-époxytrichothec-9-ène.

[Traduit par la Rédaction]

Introduction

Trichothecenes are fungal sesquiterpene mycotoxins that inhibit protein synthesis. *Myrothecium roridum*, a pathogen of melons and tomatoes, produces macrocyclic trichothecenes characterized by a bridge between C-4 and C-15 (e.g., roridin; Fig. 1). The biosynthesis of these toxins begins with the same step as that of the *Fusarium* trichothecenes T-2 toxin and 15-acetyldeoxynivalenol (Fig. 1); that is, the cyclization of farnesyl pyrophosphate to form the sesquiterpene hydrocarbon trichodiene (Fig. 1). When the P450 monooxygenase-encoding gene *Tri4* is disrupted in *Fusarium*, trichothecene production is blocked and trichodiene accumulates (Hohn et al. 1995). A previous study showed that a cluster of 3 genes in *M. roridum* has homology to 3 *Fusarium* trichothecene biosynthetic genes: *Tri4*; *Tri5*, the sesquiterpene cyclase gene for trichodiene formation; and *Tri6*, a zinc-finger regulatory gene (Trapp et al. 1998). They reported that the *M. roridum* MrTRI4 and the *Fusarium sporotrichioides* FsTRI4 proteins have 63% identity and 80% similarity over a region of 502 residues. Expression of *M. roridum Tri4 (MrTri4)* in a *F. sporotrichioides Tri4* ultraviolet (UV) mutant strain did not result in full complementation but rather in the accumulation of 2 compounds not normally observed in wild-type cultures of *F. sporotrichioides*, deoxysambucinol and 12,13-epoxytrichothec-9-ene (also called trichothecene), as well as a small amount of T-2 toxin (Trapp et al. 1998) (Fig. 1). Wild-type levels of T-2 toxin were not restored. The reaction product of MrTRI4 was not determined.

We previously determined the reaction product of FgTRI4 by expressing *Fusarium graminearum Tri4* (*FgTri4*) in *Fusarium verticillioides* behind the promoter for a fumonisin biosynthetic gene (*FUM8*) (McCormick et al. 2006*b*). *Fusarium verticillioides* has no gene homologous to *Tri4* and does not produce trichothecenes. Transgenic *F. verticillioides* expressing *FgTri4* converts exogenous trichodiene to isotrichodermin. Conversion of trichodiene to isotrichodermin requires 7 steps. Two of these steps are nonenzymatic (McCormick et al. 1990), and wild-type *F. verticillioides* can carry out the final acetylation, converting isotrichodermol to isotrichodermin. Together, the results indicated that the *F. graminearum Tri4* protein catalyzes the

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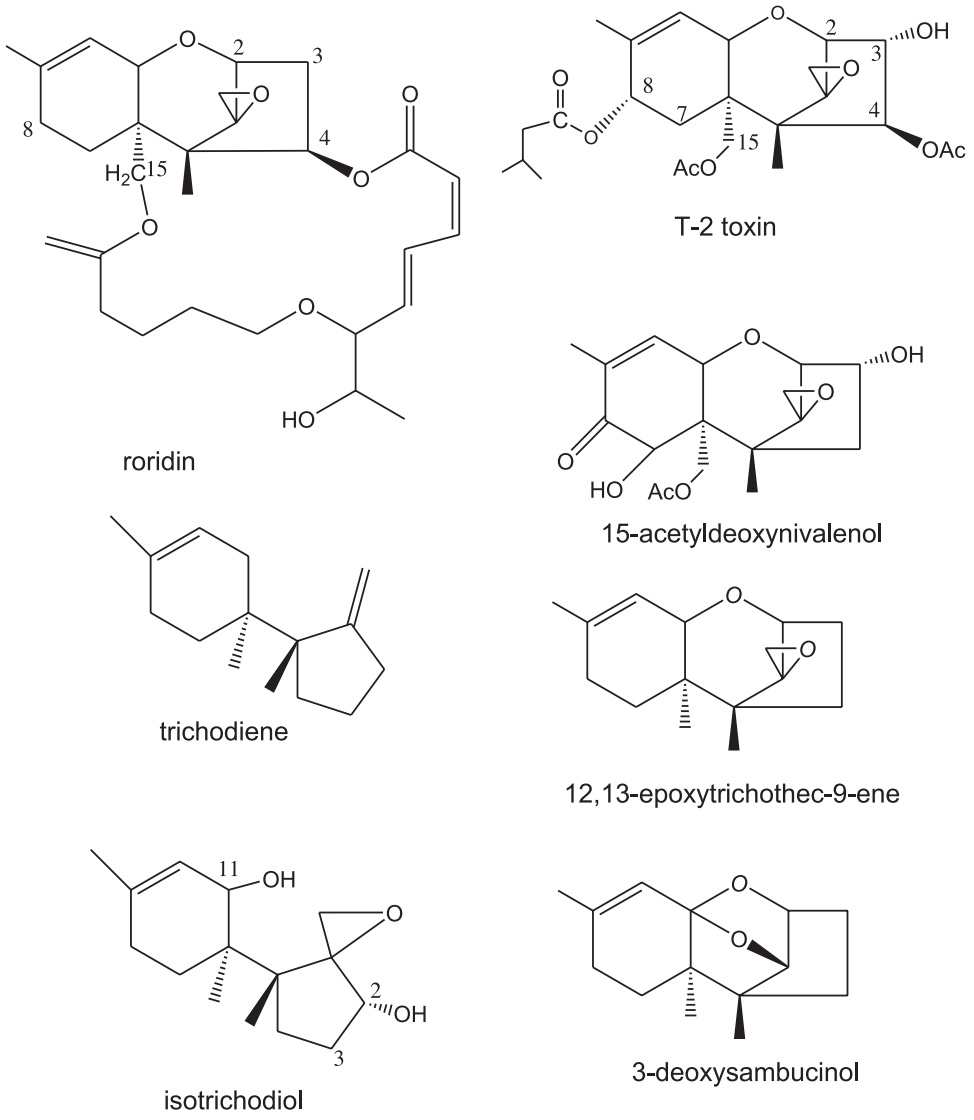
Fig. 1. Structures of trichothecenes and related compounds.



first 4 oxygenations at C-2, C-3, C-11, and C-12 required for 15-acetyldeoxynivalenol biosynthesis and that the FgTRI4 reaction product is isotrichotriol (McCormick et al. 2006*b*).

Since *M. roridum* produces macrocyclic trichothecenes that lack C-3 oxygenation, there may be fundamental differences in *Tri4* function between *Fusarium* and *Myrothecium*, and it is likely that the reaction product of MrTRI4 is not the same as the reaction product of FgTRI4. To determine the reaction product of MrTRI4, *M. roridum Tri4* was expressed in *F. verticillioides.* Transformants were grown in liquid culture and fed the TRI4 substrate trichodiene.

Materials and methods

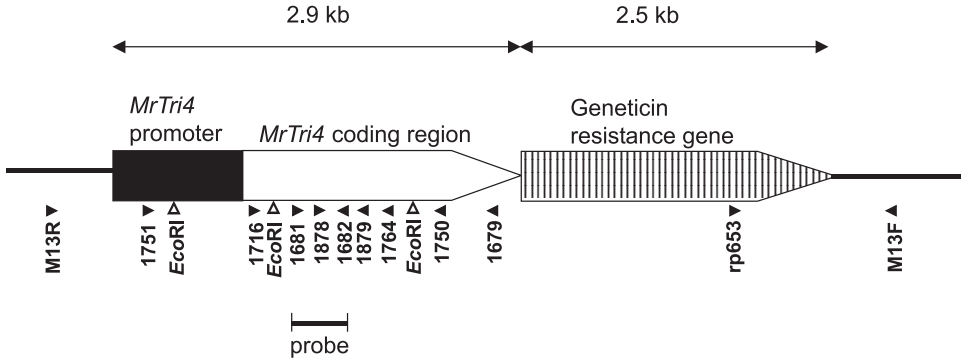
Strains

Fusarium verticillioides M-3125 is a fumonisin-producing strain (Leslie et al. 1992). Strains FvMrTri4#7-3 and FvMrTri4#7-8 are *F. verticillioides* transformants that contain the *M. roridum Tri4* gene (*MrTri4*), as described below. *Fusarium sporotrichioides* F15 is a *Tri4* disruption mutant of NRRL3299 (Hohn et al. 1995). Strains F15MrTri4#7-10 and F15MrTri4#7-15 are transformants of *F. sporotrichioides* F15 that contain the *M. roridum Tri4* gene. *Fusarium sporotrichioides* 4-4-18 (Trapp et al. 1998) is a transformant of *F. sporotrichioides* MB5493, a *Tri4*$^{-}$ UV mutant of NRRL3299 (Beremand 1987; Trapp et al. 1998). *Fusarium sporotrichioides* 4-4-18 contains the *MrTri4* gene from *M. roridum* (ATCC 52485). *Fusarium sporotrichioides* NRRL3299 is a strain producing T-2 toxin.

Media and culture conditions

All strains were grown on V8® juice agar slants or plates (Stevens 1974). Transformants (all single-spored) were grown on V8® agar slants supplemented with 1 mg/mL geneticin. Spores were stored at –80 °C in glycerol stock (50% glycerol–water, 1:1, *v*/*v*). For feeding experiments, liquid cultures of *F. verticillioides* were started with a loop from glycerol stock inoculated into 20 mL glucose – yeast extract – asparagine – malic acid medium (GYAM) (Proctor et al. 1999) or into yeast extract – peptone – dextrose (YEPD) (Ueno et al. 1975) in 50 mL Erlenmeyer flasks and

Fig. 2. Plasmid map of pMrTri4/pCR4. A 2.9 kb fragment amplified from *Myrothecium roridum* DNA containing the promoter and coding region of *MrTri4* and a 2.5 kb fragment encoding geneticin resistance were cloned into the pCR4-Blunt TOPO™ plasmid. Primer positions and orientations are indicated.



were then grown at 28 °C in the dark with orbital shaking at 200 r/min (1 r = 2πrad). *Fusarium sporotrichioides* cultures were grown on YEPD.

Trichothecenes

Trichodiene was isolated from *F. sporotrichioides* F15 (Hohn et al. 1995). Isotrichodiol standard was isolated from trichodiene-fed cultures as previously described (Hesketh et al. 1991). Isolation of 12,13-epoxytrichothec-9-ene (also called trichothecene) (Fig. 1) from liquid cultures of *F. sporotrichioides* 4-4-18 was done as previously described (Trapp et al. 1998). The identity and purity (>95%) of the compounds were confirmed by gas chromatography and low resolution mass spectrometry (GC-MS).

Chemical analyses

GC-MS measurements were made with a Model 5890 gas chromatograph (Hewlett-Packard, Palo Alto, California) fitted with a 30 m fused silica capillary column (DB-5-MS; 0.25 μm; J&W Scientific Co., Palo Alto, California). For routine screening of the trichothecene toxin phenotype, the column was held at 120 °C at injection, heated to 210 °C at 15 °C/min and held for 1 min, and then heated to 260 °C at 5 °C/min and held for 3 min.

Construction of *MrTri4* plasmids and transformants

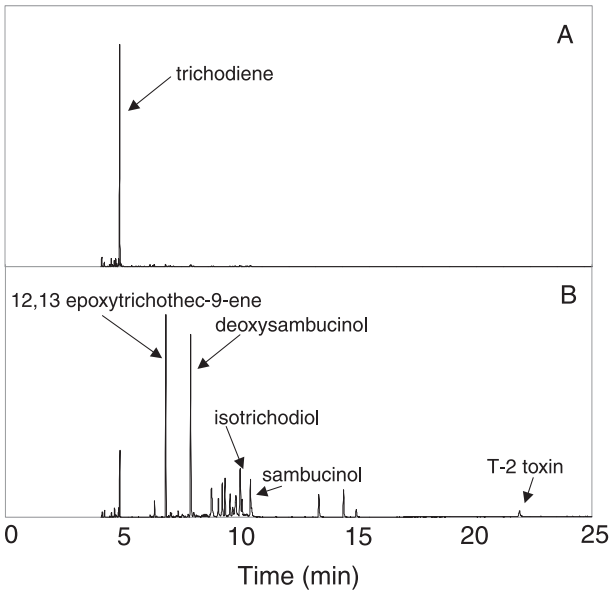
The *MrTri4* promoter and coding region were amplified using iProof® (BioRad, Hercules, California) following the manufacturer's protocol in a PTC-100 thermocycler (MJ Research, Watertown, Massachusetts) with primers 1751 (5′-CATGGTAGGGCAGCTGTC-3′) and 1679 (5′-CAGGGCGCGCCCTGAGAGCCGCTTACGGTGCGGTTTAGTGTC-3′) on a template of genomic DNA from strain 4-4-18 (Fig. 2). The resulting 2921 bp fragment was band purified (UltraClean™, MoBio, Solano Beach, California) and cloned into pCR4-Blunt TOPO™ (Invitrogen, Carlsbad, California) following the manufacturer's protocol. The cloned fragment was sequenced using an ABI Prism BigDye™ terminator cycle sequencing kit (Applied Biosystems, Foster City, California) using a Model 3700 automated DNA sequencer (Applied Biosystems) to ensure the integrity of the sequence. The plasmid was then cut with *Asc*I (underlined sequence in primer 1679) and ligated with an *Asc*I fragment carrying the selectable geneticin marker (Tanaka and Tsuge 2001; Desjardins et al. 2004). Orientation of all inserts was determined using primer pairs. M13R and M13F (Fig. 2) were obtained from sequences supplied by the manufacturer (Invitrogen). The sequence of rp653 in the geneticin gene was 5′-CTTGTCGATCAGGATGATCTGG and that of 1750 from *MrTri4* was 5′-CGGATAGACGGATAGATGGATCGTACG. Transformation of *F. verticillioides* was done as described previously (Proctor et al. 1999) with selection on a geneticin (1 mg/mL, InVivoGen, San Diego, California) containing medium. *Fusarium sporotrichioides* F15 was transformed as described previously (Proctor et al. 1997) and transformants were selected using a medium containing 1 mg/mL geneticin.

Analysis of transformants

Transformants were analyzed by both polymerase chain reaction (PCR) and Southern blotting. Genomic DNA was prepared as described previously (Hohn and Desjardins 1992) with the modification of placing the mycelial mat into liquid nitrogen immediately following harvesting by filtration. The frozen material was ground in a mortar cooled by liquid nitrogen and the powder was resuspended in extraction buffer (200 mmol/L Tris (pH 8.5), 250 mmol/L NaCl, 25 mmol/L ethylenediaminetetraacetic acid (EDTA; pH 8.0), 0.5% sodium dodecyl sulfate). After phenol–chloroform and chloroform extractions, the aqueous layer was recovered and RNase A (Qiagen, Valencia, California) added and incubated at room temperature for 15 min. DNA was precipitated overnight with 2.5 volumes of 95% ethanol, centrifuged at 12 000*g*, washed with 70% ethanol and centrifuged at 10 000*g*, and resuspended in water or TE10 (10 mmol/L Tris (pH 8.0), 0.1 mmol/L EDTA). Genomic templates were analyzed with PCR using primers 1716 (5′-ATGGCTGTCTTGAACTTCGAGACAGTCAGC) and 1764 (5′-TCTAGGCGGTGGAGATGA) that produced an 1874 bp fragment of *MrTri4*.

Southern analysis was performed on genomic DNA that had been restricted with *Eco*RI, separated on an agarose gel, transferred to Nytran SuperCharge™ membrane (Schleicher and Schuell, Keene, New Hampshire) by capillary action, and crosslinked by exposure to UV light (Stratalinker™; Stratagene, Cedar Creek, Texas). A 540 bp *MrTri4*-specific probe was made using primers 1681 (5′-CACATGAACGGCAAGGAG-3′) and 1682 (5′-GAAGAG-

Fig. 3. Gas chromatography and low resolution mass spectrometry traces of *Fusarium sporotrichioides* transformant strains (A) F15 and (B) F15MrTri4#7-10.



CTTCAAGAGTCTG-3′) labeled with [^{32}P]dCTP (Ready to Go Beads™; Amersham Biosciences, Piscataway, New Jersey) and hybridized (ULTRAhyb™; Ambion, Austin, Texas) to the membrane overnight at 42 °C. Blots were exposed to Kodak XAR™ x-ray film (Eastman Kodak Co., Rochester, New York).

Expression analysis

The *MrTri4* message was detected using reverse transcription PCR (RT–PCR). YEPD (50 mL) was inoculated with 1×10^5 spores/mL and incubated for 48 h and 200 r/min at room temperature. Total RNA was isolated from mycelia that had been harvested by filtration, immediately immersed into liquid nitrogen, and ground to a fine powder. A pencil-eraser-sized amount of powder (about 40 mg) was placed into a 2.0 mL tube containing 500 µL of acid-washed glass beads, 500 µL of AE buffer (50 mmol/L sodium acetate, 10 mmol/L EDTA (pH 8.0), 1% sodium dodecyl sulfate), and 500 µL of acid phenol (Sigma-Aldrich, St. Louis, Missouri), which had all been preheated to 65 °C. Samples were vortexed for 30 s, placed at 65 °C for 10 min with vortexing every 2 min, and then spun for 10 min at 13 000 r/min. The aqueous layer was placed into a prepared Eppendorf phase-lock tube (Fisher Scientific, Pittsburgh. Pennsylvania) and an equal volume of chloroform added. After spinning the tube for 5 min at 13 000 r/min, the aqueous layer was placed into a fresh tube and one-tenth volume of 3 mol/L sodium acetate (pH 5.2) was added, followed by the addition of an equal volume of isopropanol. The samples were spun for 30 min at 13 000 r/min and the pellet washed in 70% ethanol. After a 5 min spin at 13 000 r/min, the pellets were resuspended in water and cleaned using a RNeasy™ spin column (Qiagen). Samples were treated with DNase (Ambion) before using a One Step™ RT kit (Qiagen). *MrTri4*-specific primers 1878 (5′-TGAGGAAGATGCGTGAGGA-3′) and 1879 (5′-TGGTGTCGTACAGCTCGAAGT-3′) were used in the amplification step. Because these primers span 2 introns, they produce a 662 bp fragment from genomic DNA but a 521 bp fragment from cDNA.

Whole-cell feeding

GYAM or YEPD cultures (20 mL in 50 mL Erlenmeyer flasks) of *F. verticillioides* M-3125 and *FvMrTri4* mutant strains were started with a loop of spores from a glycerol stock suspension of spores. After 24 h, an acetone solution of trichodiene was added to give a final concentration of 368 µmol/L. The final concentration of acetone in the cultures was less than 1%. Aliquots (5 mL) were removed and extracted with 2 mL ethyl acetate and analyzed by GC-MS at time points up to 7 days and examined for the appearance of oxygenated products. To conduct spectral analysis of the 12,13-epoxytrichothec-9-ene formed, larger-scale cultures (100 mL in 250 mL Erlenmeyer flasks) of selected transformants were grown and amended with 15 mg of trichodiene dissolved in 400 µL of acetone.

Results

MrTri4 expression in F15

The *MrTri4* expression vector consisted of the *MrTri4* coding region and 3′-flanking DNA and *MrTri4* promoter (Fig. 2). *Fusarium sporotrichioides* F15 produced trichodiene (retention time = 6 min) in liquid culture (Fig. 3A). Following transformation of the *MrTri4* expression vector into *F. sporotrichioides* F15, 50 geneticin-resistant transformants were selected and grown in liquid YEPD culture. Three transformants did not have an altered phenotype and accumulated trichodiene. The remaining transformants converted the trichodiene to oxygenated metabolites (Fig. 3B). Although there was some variation in the amounts of each compound, a mixture of 3-deoxysambucinol, sambucinol, isotrichodiol, and 12,13-epoxytrichothec-9-ene was usually produced. Most transformants that made oxygenated products also made small but detectable amounts of T-2 toxin.

MrTri4 expression in *F. verticillioides*

Following transformation of the *MrTri4* expression vector into *F. verticillioides* M-3125, 81 geneticin-resistant transformants were recovered and initially screened on GYAM for their ability to metabolize trichodiene added to liquid cultures. In cultures of the transformants that metabolized trichodiene, a small amount of 12,13-epoxytrichothec-9-ene (Fig. 1) was detected in cultures of trichodiene-fed transformants but several other compounds tentatively identified as apotrichothecenes were also produced. The toxin profile was simpler to interpret when these transformants were grown on YEPD medium. A time-course experiment showed that as trichodiene concentrations decreased, isotrichodiol concentrations increased (Fig. 4). After 4 days, 12,13-epoxytrichothec-9-ene (Fig. 4) began to accumulate.

PCR and Southern analysis of mutant strains

Sequencing analysis indicated that no nucleotide changes were introduced during the cloning steps. PCR analysis of 4 randomly selected *F. verticillioides* and *F. sporotrichioides* transformants that could metabolize trichodiene, using 2 primers in the *MrTri4* sequence, showed the presence of a

Fig. 4. Gas chromatography and low resolution mass spectrometry traces showing the time course of *Fusarium verticillioides* transformant FvMrTri4#7-8 after being fed trichodiene.

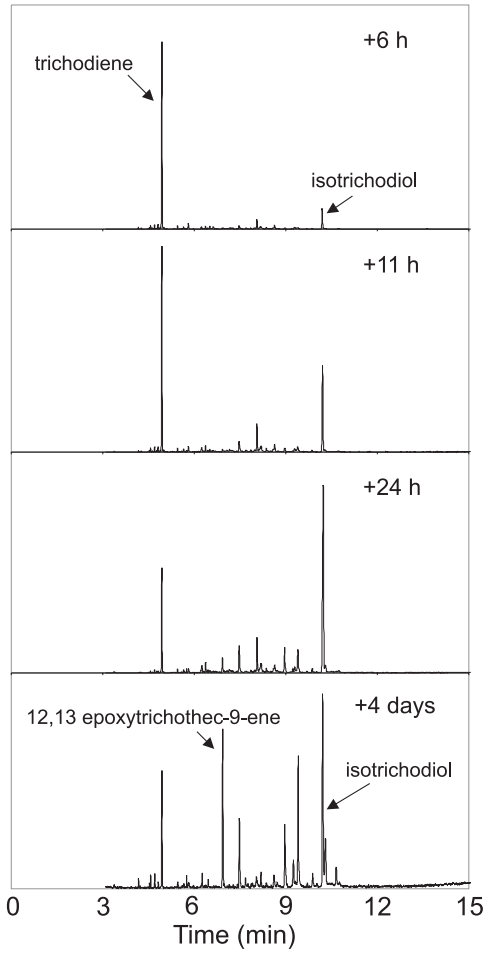
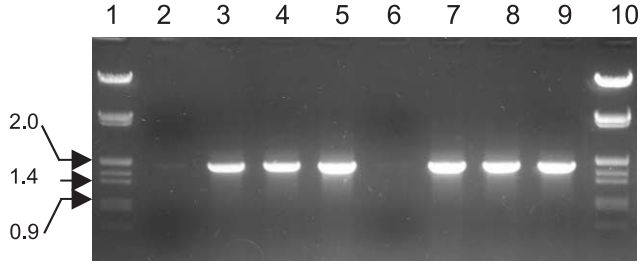


Fig. 5. Polymerase chain reaction analysis of wild-type and transformant strains. Primer pair 1716–1764 was used to determine the presence of *MrTri4* sequence (expected size of 1874 bp). Lanes 1 and 10, λ DNA restricted with *Hin*dIII–*Eco*RI, markers indicate kilobases; lane 2, wild-type *Fusarium verticillioides* M-3125; lane 3, transformant FvMrTri4#7-3; lane 4, transformant FvMrTri4#7-8; lanes 5 and 9, *Fusarium sporotrichioides* 4-4-18; lane 6, *F. sporotrichioides* F15; lane 7, transformant F15MrTri4#7-10; lane 8, transformant F15MrTri4#7-15.



band (about 1874 bp) (Fig. 5, lanes 3, 4, 7, and 8) corresponding to the expected size of *MrTri4* from 4-4-18 (Fig. 5, lanes 5 and 9). There was no such band detected in either *F. verticillioides* M-3125 (Fig. 5, lane 2) or *F. sporotrichioides* F15 (Fig. 5, lane 6), indicating that the

Fig. 6. Southern analysis of wild-type and transformant strains. Genomic DNA was restricted with *Eco*RI. Lane 1, *Fusarium verticillioides* M-3125; lane 2, transformant FvMrTri4#7-3; lane 3, transformant FvMrTri4#7-8; lane 4, *Fusarium sporotrichioides* F15; lane 5, transformant F15MrTri4#7-10; lane 6, transformant F15MrTri4#7-15. Markers indicate kilobases.

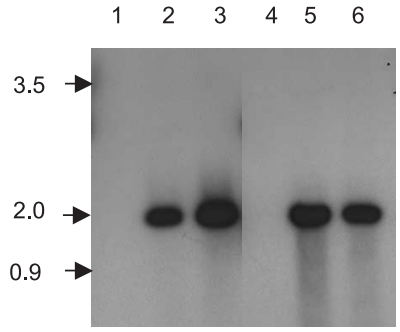


Fig. 7. Reverse transcriptase – polymerase chain reaction analysis of wild-type and transformant strains. Primer pair 1878–1879 spans 2 introns in the *MrTri4* sequence. Lanes 2, 4, 6, and 8 had cDNA as template; lanes 3, 5, 7, and 9 had genomic DNA as template. (A) Analysis of *Fusarium verticillioides* wild type and transformants. Lane 1, λ DNA restricted with *Hin*dIII–*Eco*RI; lanes 2 and 3, *F. verticillioides* M-3125; lanes 4 and 5, transformant FvMrTri4#7-3; lanes 6 and 7, transformant FvMrTri4#7-8; lanes 8 and 9, *Fusarium sporotrichioides* 4-4-18. (B) Analysis of *F. sporotrichioides* F15 and transformants. Lane 1, λ DNA restricted with *Hin*dIII–*Eco*RI; lanes 2 and 3, *F. sporotrichioides* F15; lanes 4 and 5, transformant F15MrTri4#7-10; lanes 6 and 7, transformant F15MrTri4#7-15; lanes 8 and 9, *F. sporotrichioides* 4-4-18. Standard markers are in kilobases.

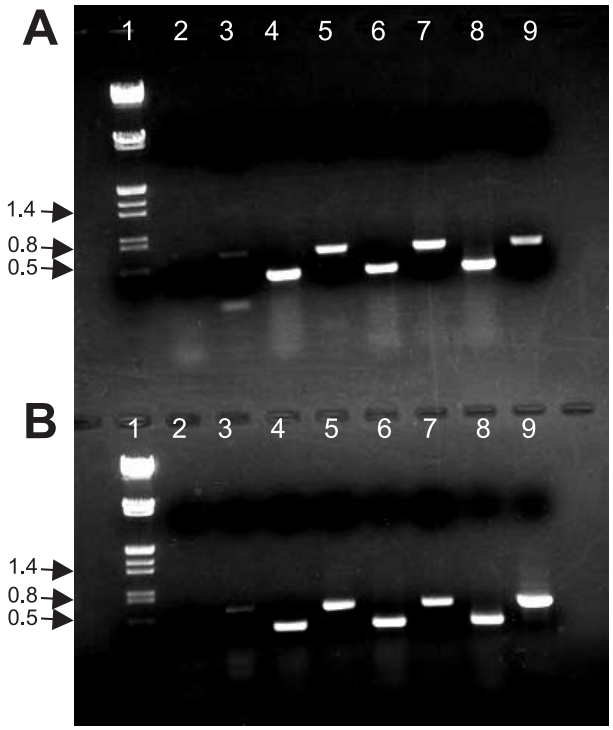
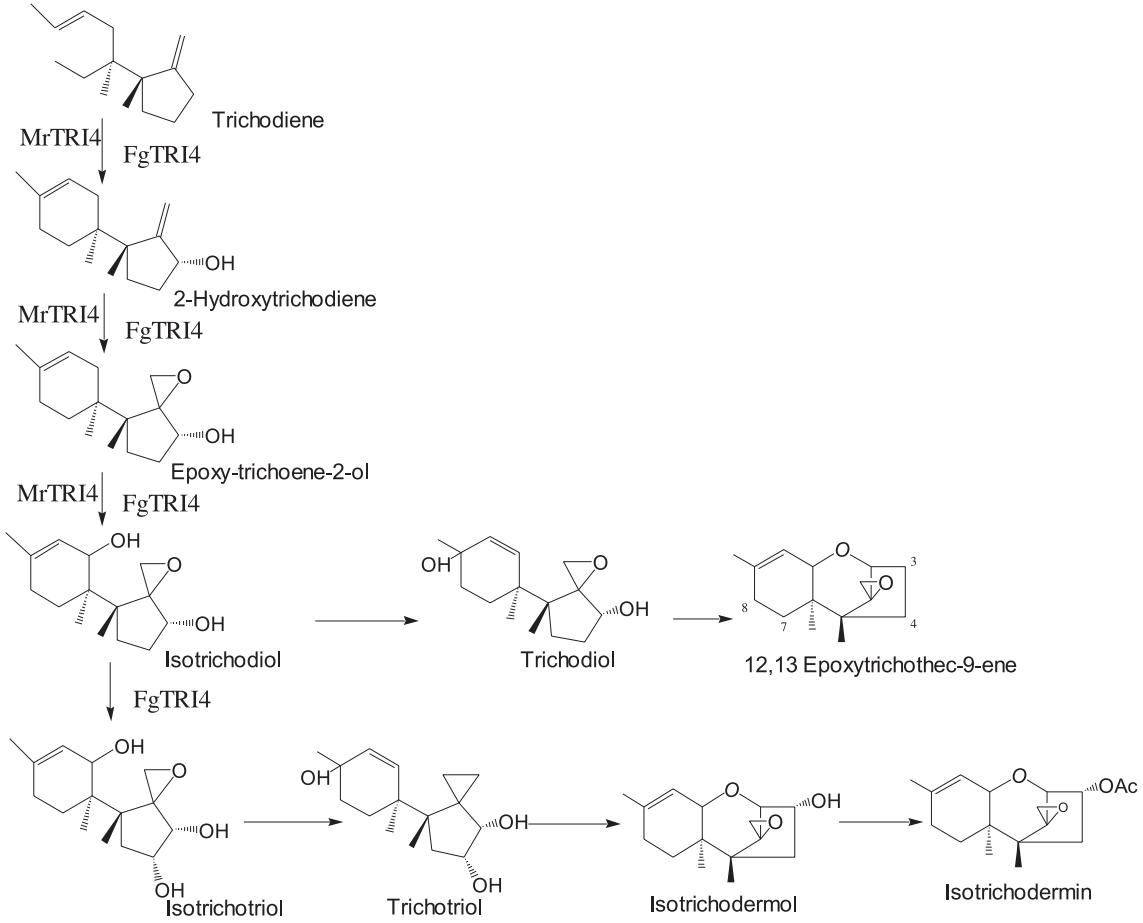


Fig. 8. Pathway for early trichothecene metabolites in *Myrothecium* sp. and *Fusarium* sp. showing biosynthetic steps catalyzed by MrTRI4 or FgTRI4.



band in transformant strains was from the introduced sequence.

Southern analysis also confirmed the presence of *MrTri4* sequence in 4 selected transformants (Fig. 6, lanes 2, 3, 5, and 6). Restriction of the genomic DNA with *Eco*RI followed by probing with a radiolabeled fragment produced the expected 2.0 kb band (see Fig. 2 for restriction sites and location of the probe). No sequence homologous to *MrTri4* was found in *F. verticillioides* M-3125 (Fig. 6, lane 1) or in *F. sporotrichioides* F15 (Fig. 6, lane 4).

Expression analysis

Expression of the cloned *MrTri4* gene in either *F. verticillioides* M-3125 or *F. sporotrichioides* F15 was analyzed by RT–PCR. Primers that span 2 introns were chosen to confirm that the DNase I effectively eliminated any contaminating genomic DNA from the RNA preparation. Any genomic DNA that was present would produce a 662 bp fragment while the cDNA would produce a 521 bp fragment (Fig. 7). Transformants of *F. verticillioides* M-3125 (FvMrTri4#7-3 and FvMrTri4#7-8) showed a 521 bp fragment formed from the RNA (Fig. 7A, lanes 4 and 6), while the genomic DNA produced a 662 bp fragment (Fig. 7A, lanes 5 and 7). The same bands were present in *F. sporotrichioides* 4-4-18, which carries the wild-type *MrTri4* (Fig. 7A, lanes 8 and 9). No corresponding *MrTri4* band was found in RNA from cultures of wild-type *F. verticillioides* M-3125 (Fig. 7A, lanes 2 and 3).

Transformants of *F. sporotrichioides* F15, F15MrTri4#7-10, and F15MrTri4#7-15 also performed in the same fashion, since the mRNA produced a band of 521 bp (Fig. 7B, lanes 4 and 6) and the DNA produced a band of 662 bp (Fig. 7B, lanes 5 and 7). No bands appeared in the RNA or DNA from *F. verticillioides* M-3125 (Fig. 7B, lanes 2 and 3), while the band was present in *F. sporotrichioides* 4-4-18 (Fig. 7B, lanes 8 and 9).

Discussion

Our results indicate that MrTRI4 converts trichodiene to isotrichodiol. *Myrothecium roridum* P450 gene *Tri4* controls 3 oxygenations at C-2, C-11, and C-12 in contrast to *F. graminearum Tri4* that controls 4 oxygenations at C-2, C-11, C-12, and C-3 (Fig. 8). Sequence analysis indicates that *F. graminearum Tri4* and *M. roridum Tri4* have 66.9% identity. In comparison, there is 86% identity between the *Tri4* sequence in 2 trichothecene-producing species, *F. graminearum* and *F. sporotrichioides.*

Fusarium sporotrichioides MB5493 is a *Tri4* UV mutant strain derived from NRRL3299 that makes no T-2 toxin but accumulates trichodiene (McCormick et al. 1989; Beremand and McCormick 1992). Expression of *F. sporotrichioides*

Tri4 in MB5493 restores normal production of 200–300 μg/mL T-2 toxin (Hohn et al. 1995). When *M. roridum Tri4* (*MrTri4*) was expressed in *F. sporotrichioides* MB5493, transformants expressing *MrTri4* did not have normal T-2 toxin production but rather accumulated isotrichodiol, 12,13-epoxytrichothec-9-ene, sambucinol, and deoxysambucinol. *MrTri4* transformants produce only 10 μg/mL T-2 toxin (Trapp et al. 1998). The fact that production of trichothecenes is restored in transformants leads to the conclusion that there is complementation and that MrTRI4 and FsTRI4 perform related functions in trichothecene biosynthesis (Trapp et al. 1998).

The present study confirms that there is a partial complementation of *Fusarium Tri4* with *Myrothecium Tri4*. *MrTri4* is expressed in the *F. sporotrichioides Tri4* disruption mutant, strain F15, and transformants produce a small amount of T-2 toxin and larger amounts of deoxysambucinol, sambucinol, isotrichodiol, and 12,13-epoxytrichothec-9-ene (Fig. 3B). Sambucinol and deoxysambucinol are shunt products of isotrichodiol (Mohr et al. 1984). The likely cyclization product of isotrichodiol is 12,13-epoxytrichothec-9-ene. It is not clear why even a small amount of T-2 toxin is produced.

To determine the specific product of MrTRI4 oxygenation, *M. roridum Tri4* was expressed in *F. verticillioides* under the control of its own promoter. *Fusarium verticillioides* is a fumonisin producer and makes no trichothecenes, but it has been shown to be a good host for heterologous expression of trichothecene P450 genes (McCormick et al. 2006*a*, 2006*b*). In previous studies, *F. graminearum Tri4*, *F. graminearum Tri1*, and *F. sporotrichioides Tri1* were expressed in *F. verticillioides* behind the promoter of a fumonisin gene, *FUM8* (McCormick et al. 2006*a*, 2006*b*). Expression of *MrTri4* behind the *FUM8* promoter in *Escherichia coli* resulted in the generation of a lethal factor (unpublished results). For this reason, in this study the *MrTri4* was expressed in *F. verticillioides* behind the *MrTri4* promoter. The success of this construction shows that a fumonisin gene promoter is not required for expression of trichothecene genes in *F. verticillioides*. In addition, fumonisin liquid culture conditions were found to be unnecessary. YEPD medium has been routinely used for trichothecene production (Ueno et al. 1975) and has a higher pH than the fumonisin production medium GYAM. Oxygenation products of trichodiene can be obtained on both GYAM and YEPD media. Since some trichothecenes are susceptible to chemical rearrangements in acidic conditions, YEPD may be preferable for feeding studies.

The results of this study indicate that liquid cultures of *F. verticillioides* mutants carrying *MrTri4* convert trichodiene to isotrichodiol (Fig. 4). The isotrichodiol formed by liquid cultures of *FvMrTri4* transformants are then likely nonenzymatically converted to 12,13-epoxytrichothec-9-ene. Previous work has shown that trichodiene is not metabolized by cultures of *F. verticillioides* or by incubation in the culture medium, but that isotrichodiol is converted to 12,13-epoxytrichothec-9-ene after incubation in GYAM medium (pH 2.6) (McCormick et al. 2006*b*). This is analogous to the nonenzymatic conversion of isotrichotriol to trichotriol and of trichotriol to isotrichodermol (McCormick et al. 1989). In *Fusarium*, 12,13-epoxytrichothec-9-ene is a dead-end metabolite. T-2 toxin biosynthesis requires C-3 oxygenation prior to both cyclization and C-15 oxygenation (McCormick et al. 1990).

Myrothecium and *Trichothecium* species produce trichothecenes that lack C-3 oxygenation and likely share the early pathway with the 3 initial oxygenation steps controlled by *Tri4*, cyclization to form 12,13-epoxytrichothec-9-ene, and subsequent oxygenations at C4, C-8, or C15. The present results show that the branch leading to macrocyclic trichothecenes is at *Tri4*. We previously reported that there are also 2 forms of *Tri1*. In *F. graminearum*, *Tri1* controls both C-7 and C-8 hydroxylation in deoxynivalenol biosynthesis (McCormick et al. 2006*b*) and the *F. sporotrichioides Tri1* homolog controls only C-8 hydroxylation in T-2 toxin biosynthesis. Studies of the protein crystal structure may shed light on the differences between homologous proteins in trichothecene biosynthesis.

In conclusion, *MrTri4* encoded a multifunctional oxygenase required for the first 3 steps of macrocyclic trichothecene biosynthesis in *M. roridum*. This is in contrast to *Fusarium Tri4* that controls 4 oxygenation steps. This study also showed that fumonisin gene promoters are not required for *F. verticillioides* expression of trichothecene genes.

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